ETHYLENEDIAMINE TETRAACETIC ACID INHIBITION OF ALKALINE PHOSPHATASE FROM NORMAL AND LEUKEMIC TISSUES OF C57BL MICE

A THESIS

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

BY

MOHAMMED YAHYA KHWAJA

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

MAY 1974

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I wish to extend my sincere thanks to Dr. Judith Rae Lumb, who has helped and guided me during the entire course of this research.

I also wish to thank Dr. J. M. Browne, Associate Professor and Dr. Winfred Harris, Professor of Biology, Clark College for reviewing the manuscript and for their constructive criticism.

Finally I wish to gratefully acknowledge receipt of the letters of encouragement from my parents in Pakistan, who elevated my spirits constantly.

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Alkaline phosphatase (APase) has been associated with the thymic lymphoma by several workers. Smith (1961, 1962) has shown the correlation between the presence of APase and thymic lymphoma development in C57Bl/6 and AKR strains of mice. Metcalf et al. (1962) observed the same kind of relationship in C3H and AKR strains of mice. Lagerlöf and Kaplan (1967), using histochemical methods, showed that APase appears concurrently with the development of thymic lymphoma. Lumb and Doell (1970) have compared APase from thymic lymphomas, normal adult spleen and 16-day-old embryonic thymus and found them to be related on the basis of biochemical parameters. On the basis of this observation they suggested that APase is a cell-coded enzyme which is derepressed in the course of malignant transformation.

This study was undertaken to further characterize the APase from normal tissues and leukemic tissues and to see if the APase of thymic lymphoma is a derepressed enzyme as has been suggested by Lumb and Doell (1970). For this purpose the parameters of inhibition and metallic ion activation were used to compare the APase from normal and leukemic tissues.

Inhibitors have been used for a long time in the iden-

tification of tissue sources of APase. Bodansky (1937) has used bile acids in the differentiation of APases from bone, kidney, intestine, and serum. Fishman et al. (1963) used L-phenylalanine as an organ specific, stereospecific inhibitor of intestinal APase from rat and human intestine. Cox and Griffin (1967) studying the physical and chemical properties of APase from mammalian tissues, cell cultures and E. coli reported that these various preparations of APase differ from each other in respect to the concentration of zinc and L-phenylalanine required to inhibit the enzymatic activity. Ethylenediamine tetraacetic acid has been used as an inhibitor of APase by Clark and Porteous (1965). APase from the rabbit intestine, when dialyzed against EDTA, can be reactivated by the addition of cobalt alone or magnesium and zinc ions together, neither magnesium nor zinc was effective alone (Clark and Porteous, 1965). The important observation was that zinc should not exceed an optimum limit.

Preliminary studies by Williams (1971) have indicated that APase from thymic lymphomas is inhibited by zinc, L-phenylalanine and EDTA, and that EDTA inhibition is reversed by the addition of magnesium. These studies were undertaken to compare the APase from normal tissues with that from leukemic tissues utilizing the above parameters.

CHAPTER II

REVIEW OF LITERATURE

Mouse Leukemia Viruses

As a result of its similarity to the human disease, murine leukemia has been of significant experimental interest for many years. The demonstration by Gross (1951) that a cell free extract from a spontaneously produced lymphoma would produce leukemia in other mice refocused attention on this experimental approach to an understanding of the mechanism of neoplasia.

Lieberman and Kaplan (1959) reported the leukemogenic activity of a cell free filtrate from a radiation-induced C57Bl mouse lymphoma. They observed that even non-irradiated thymic implants in irradiated hosts would develop tumors. This phenomenon of indirect induction led them to believe that a latent sub-cellular agent analogous to the filtrable agent of spontaneous mouse leukemia reported by Gross (1951) is activated by irradiation and causes malignant transformation in susceptible lymphoid cells. Their results provided the first evidence for directly linking the external carcinogens to viruses. Haran-Ghera (1967) induced generalized lymphatic leukemia in C57Bl mice with 7,12-dimethylbenz(a)anthracene. When filtrates from these chemically induced lymphomas were injected into the thymic implants in thymectomized, irradiated adult mice, 15-27 per-

cent developed a generalized type of lymphatic leukemia. This report described for the first time the successful induction of lymphatic leukemia in adult C57Bl mice. Doell and Mathieson (1970) showed the induction of thymic lymphomas in C57Bl mice and W/Fu rats injected intrathymically during neonatal life with a virus later named C57LV (Doell and Mathieson, 1971). This virus was isolated from 6-mercaptopurine-induced thymic lymphoma and maintained through serial passages. Ferrer and Kaplan (1968) have shown by antigenic analysis that the virus induced by irradiation (RadLV) is related to Gross type virus. Doell and Mathieson (1971) have also shown the same kind of relationship between Gross virus, RadLV and C57LV utilizing the immunofluorescence test.

Role of APase in Mouse Leukemia

Smith (1961, 1962) has utilized histochemical techniques in the study of thymuses of AKR and irradiated C57Bl/6 strains of mice and has shown the association of APase with the thymic lymphomas in both of these strains of mice. Her studies indicated a lower incidence of positive reactive cells in the thigh-shielded mice. Kaplan and Brown (1952) have also shown a lower incidence of thymic lymphoma development in the thigh-shielded mice. Smith (1962) therefore correlated the appearance of APase with the development of lymphoma. Her histochemical results emphasized the

correlation between sites of reactivity in the thymus and the development of induced lymphoma. In the initial stages after irradiation when the lobes looked normal there were clusters of positive cells among negative lymphocytes. She suggested that these histochemical changes might precede the histological changes. Metcalf et al. (1962) have studied the APase activity of mouse lymphoma tissue from the AKR mice, (leukemia incidence 90%) and from the low leukemic C3H strain of mice (leukemia incidence 5%). They found that APase activity of the thymic lymphoma is higher as compared with the normal thymic tissues. According to one of their histochemical observations, the thymic lymphoma APase is associated with the lymphocytes. They also found higher APase activity in preleukemic thymuses, but since the activity could not be detected in the lymphocytes, they concluded that APase activity appears in the lymphocytes only after the cells have become frankly neoplastic. On the whole their results did not justify the conclusion that higher content of APase activity in the lymphoma tissue is a necessary part of the neoplastic transformation. Siegler and Rich (1967) have studied the significance of increased APase activity in thymic lymphomas. The Gomori histochemical technique which they used for the demonstration of APase activity did not show APase in the early stages of lymphoma development. The lack of sensitivity of the staining tech-

nique for APase might have been responsible for the negative results. Lagerlöf and Kaplan (1967) have demonstrated by the more sensitive azo dye technique that APase is associated with the neoplastic cells and is formed concurrent with neoplastic transformation. They performed different experiments such as adrenalectomy, thymectomy and irradiation of mice with shielded thighs to rule out a connection between APase and non-malignant cell proliferation. Lumb and Doell (1970) using the biochemical parameters have shown that APase of thymic lymphoma resembles normal adult spleen and 16-day-old embryonic thymus APase activities. They postulated that APase of thymic lymphomas appears due to the derepression of embryonic functions.

Use of Inhibitors in the Identification of APase Isoenzymes

Inhibitors have been used for a long time in the identification of tissues sources of APase isoenzymes. Cloetens (1939) and Drill and Riggs (1946), utilizing cyanide inhibition in the identification, showed two types of APase in animal tissues. Bodansky (1937) used bile acids in distinguishing the APase from bone, kidney, intestine, and serum. Fishman et al. (1962), while evaluating the effect of 130 compounds on the APases from different rat tissues, found that L-phenylalanine preferentially inhibited intestinal APase. Zinc sulfate inhibited bone, liver, lung and kidney

APase, but not intestinal or blood serum APase. Cox and Griffin (1967) have studied the various physical and chemical properties of APases from mammalian tissues, cell cultures and E. coli and reported that these various enzyme preparations differ from one another with respect to the concentration of zinc sulfate and L-phenylalanine required to inhibit their enzymatic activities. Eaton and Moss (1967), while studying the orthophosphatase and pyrophosphatase activities of human intestinal and liver APases, found that L-phenylalanine inhibited both the activities of intestinal APase but did not affect either of the activities of liver APase. Rufo and Fishman (1972) used L-Homoarginine, a specific inhibitor of liver type APase, to demonstrate the presence of APase in the rat intestine. Belle (1972) used a number of inhibitors in the identification of APases from several tissues of the dog. The results showed that of the various inhibitors used including EDTA, L-phenylalanine, KCN, potassium cyanide and L-cysteine, only levamisole had a differentiating effect and could be used in the identification of canine intestinal APase.

CHAPTER III

MATERIALS AND METHODS

Normal Tissues

The normal spleen, placenta and embryo were obtained from young male and 16-day-pregnant female C57Bl mice respectively.

Tumors

Tumor numbers H-98, U-1 and H-36 were obtained from mice which had been injected intrathymically during neonatal life with C57LV in its fourteenth passage (Doell and Mathieson, 1970). H-58 was a 6-mercaptopurine-induced tumor; H-117 was a spontaneous tumor; H-208 and H-253 were obtained by transplanting cells from virally induced tumors.

Buffers

Ammediol buffer (2-amino-2methyl-1,3-propanediol) was used in all the experiments at pH 10 at 37 C in a final concentration of 0.04 M. In all cases pH was adjusted by employing 1 N HCl and 1N NaOH. In the case of L-phenylalanine inhibition and EDTA inhibition and its reversal with magnesium ion, the appropriate amounts of these were added to the buffers before adjusting the final pH to 10. Tris (hydroxymethyl) aminomethane at pH 7 in a final concentration of 0.1 M was used in the determination of magnesium content of tissue extracts.

Substrate

Para-nitrophenyl phosphate (p-NPP) at a final concentration of 3.38 mM was used as a substrate in all the experiments.

Inhibitors

Zinc sulfate, L-phenylalanine and ethylenediamine tetraacetic acid (EDTA), were used as inhibitors of APase. Zinc sulfate was used at a final concentration of 0.01 mM, L-phenylalanine at a final concentration of 10.0 mM and EDTA at final concentrations of 1.0 mM and 0.1 mM.

Metal Ion Activator

Magnesium chloride in final concentrations of 5.0 mM and 10.0 mM was used as an activator of APase.

Preparation of Enzyme Extract

Tissues which have been previously frozen or, in some cases, fresh tissues obtained from a sacrificed mouse were used in these experiments. A small amount of tissue was transferred into about 2 ml of 0.015 M NaCl solution on a petri dish on ice. The tissue was minced with clean scissors and then transferred into a glass homogenizer. Five ml of 0.015 M NaCl solution was added to the glass homogenizer containing minced tissue. The tissue was homogenized thoroughly in an ice bath and then passed through three or four layers of cheese cloth into another clean tube. The extracts were dispensed into small tubes and stored at -4 C.

p-NPP Assay

The assay was performed in a one-milliliter reaction volume. The reaction mixture contained 0.8 ml Ammediol buffer at pH 10.0, 0.1 ml enzyme extract and 0.1 ml p-NPP in a final concentration of 3.38 mM. The reaction was carried out at 37 C and stopped after 30 minutes by adding 2 ml 0.5 N NaOH containing 0.1 M EDTA. The concentration of p-nitrophenol was determined by reading the absorbance at 400 nm and referring to a calibration curve. Blanks were prepared in a similar way except that EDTA in NaOH was added before adding substrate. The average of three experimental tubes was calculated and the blank was subtracted from the average reading.

Inhibition Experiments

Inhibition experiments were performed in the presence of EDTA, zinc sulfate or L-phenylalanine, while controls were run without any inhibitor. Assays were run as has been indicated and the results were compared to control. The percentage of inhibition was calculated with formula:

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100$$

where "A" is the activity in the absence of any inhibitor, and "B" is the activity in the presence of an appropriate concentration of an inhibitor.

EDTA Experiments

The inhibition experiments were run in the presence of 1 mM and 0.1 mM EDTA. The inhibition with each of these concentrations was reversed by 5.0 mM and 10.0 mM magnesium. The controls for each of these concentrations were run simultaneously and the percentage of control was calculated.

Magnesium Content of Tissue Extracts

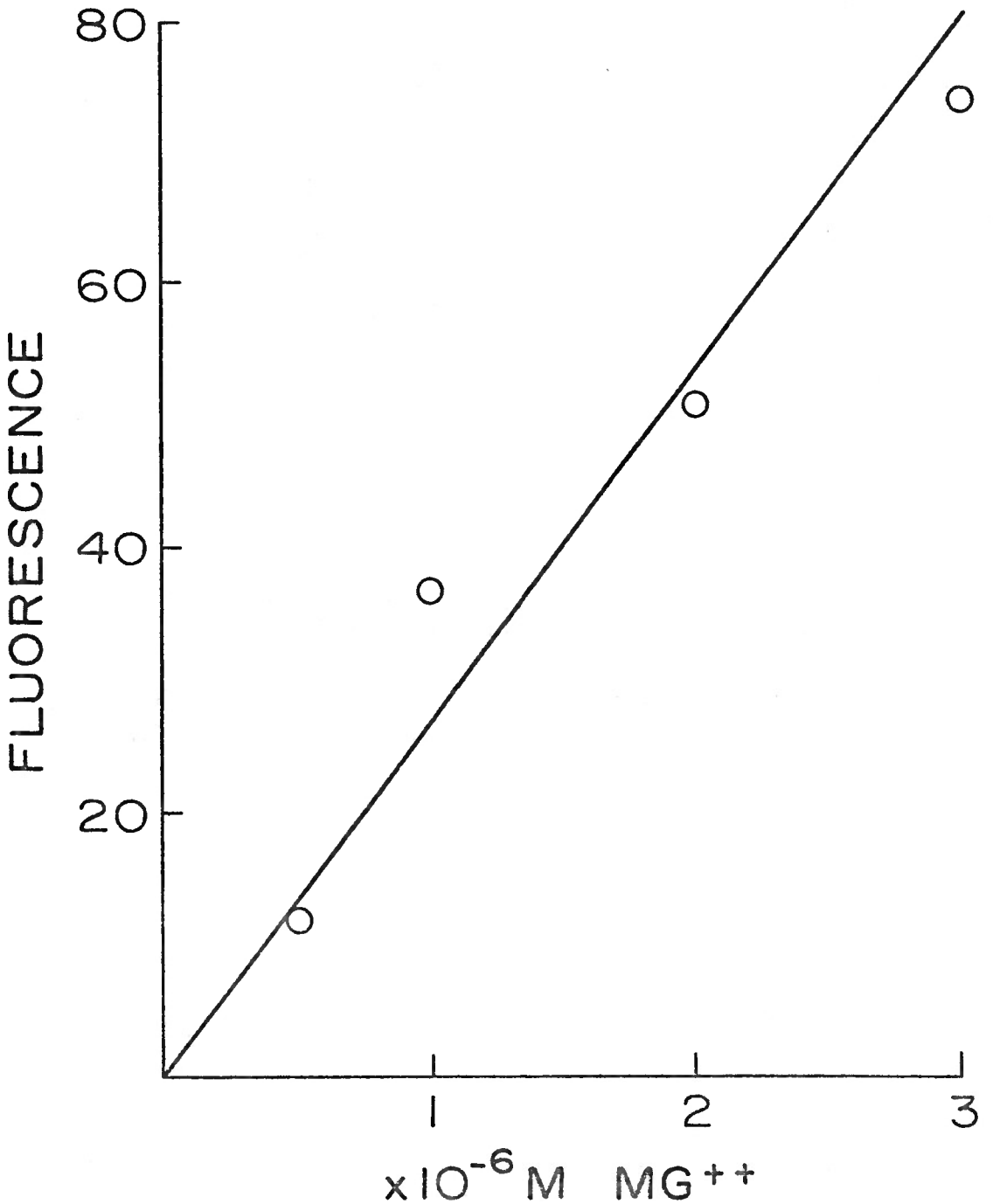
Magnesium ions are chelated by 8-hydroxy-5-quinoline sulfonic acid. The resulting chelate fluoresces at 500 nm when excited by the light of 360 nm. There is little interference from chelates of other heavy metals (Schachter, 1959, 1961). The working reagent included final concentrations of buffer and 8-hydroxy-5-quinoline sulfonic acid of 0.1 M and 0.0025 M respectively. The fluorescence spectrophotometer was warmed for an hour before use. It was adjusted to read "0" on the scale with 4 ml of buffer and reagent. Then one lambda of tissue extract was added and the reading recorded. The concentrations of standards used included 0.5×10^{-6} M, 1.0×10^{-6} M, 2×10^{-6} M and 3×10^{-6} M concentrations of magnesium. The standard dilutions of magnesium were read by zeroing with 3.9 ml of buffer and reagent solution and adding 0.1 ml of the standard dilution. Each of the standard dilutions was read twice. The average values were calculated for standards and tissue extracts. The magnesium content of each tissue

was calculated by referring to the standard curve (Fig. 1).

Statistics

A t-test analysis of variance procedure was used to find out whether there was a significant difference between APase activities of leukemic and normal tissues (Goldstein, 1964). The data were computed at the Atlanta University Center Computing Center using the TTSTT subroutine of the Scientific Subroutine Package with an IBM (International Business Machines) 1130 computer.

Fig. 1. Standard curve for the determination of magnesium content. Fluorescence spectrophotometry was used with chelating agent 8-hydroxy-5-quinoline sulfonic acid in 0.0025 M final concentration and tris (hydroxymethyl) aminomethane in a final concentration of 0.1 M at pH 7.0.


80
60
40
20
FLUORESCENCE
1
2
3
x10-6 M MG++

CHAPTER IV

EXPERIMENTAL RESULTS

Inhibition

Figure 2 shows the results obtained with the use of zinc sulfate at a final concentration of 0.01 mM. There was a statistically significant difference between APase from the embryo and placenta and that from spleen and leukemic tissues ($p < 0.01$ by t test, Table 1). Embryonic APase is inhibited up to 32% while placental APase is inhibited up to 56%. Neither leukemic APase activities nor spleen APase activities are affected at this concentration.

Figure 3 shows the inhibitory effect of 10.0 mM concentration of L-phenylalanine. The APase activities from the normal as well as from the leukemic tissues are partially inhibited at this concentration of L-phenylalanine. There was no statistically significant difference between the APase isoenzymes of normal and leukemic tissues ($p > 0.01$, Table 1).

Magnesium Ion Activation

Figure 4 shows the effect of magnesium ions on individual lymphoma APase activities and the average of tumor values. All of them are activated except H-253 which does not show any significant activation. The other tumors are activated to varying extents.

Fig. 2. Inhibitory effect of zinc sulfate on the alkaline phosphatase activity of the leukemic tissues and the normal tissues. H-117 was a spontaneous tumor, U-1 and H-36 were viral induced tumors. The normal tissues included were adult spleen (S), 16-day embryo (E), and 16-day placenta (P). Zinc sulfate was used in 0.01 mM final concentration. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. Percent inhibition is indicated by the bar. The range of values is shown (⊢——⊣).

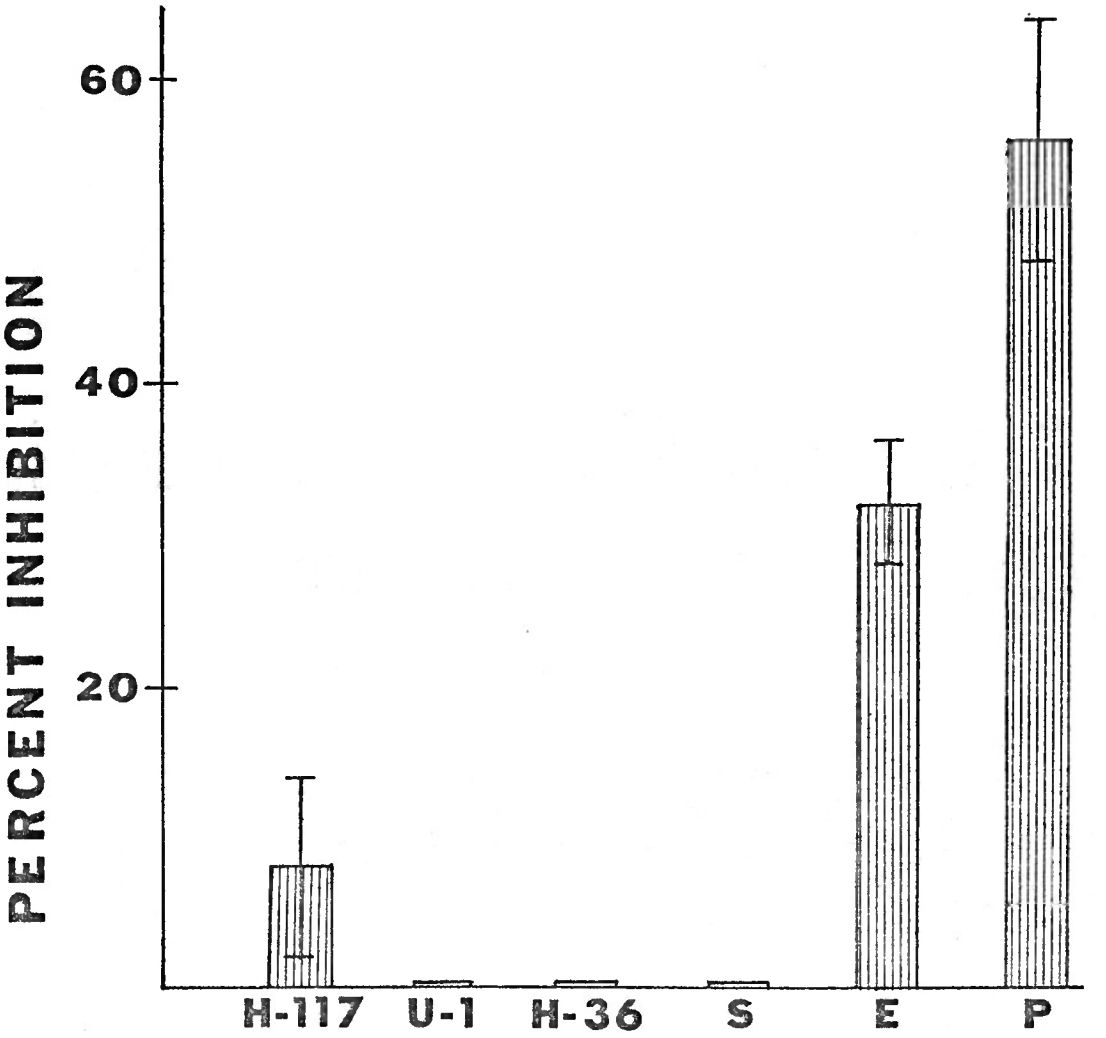

PERCENT INHIBITION
60
40
20
H-117
U-1
H-36
S
E
P

TABLE I

Statistical Comparison of Alkaline Phosphatase of Normal and Leukemic Tissues Using Student's t-Test

Characteristic	Tissues compared	Calculated t value	t Value for p=0.01*
Zinc Inhibition	Lymphoma versus Spleen	-0.207 (6)**	3.71 (6)
	Lymphoma versus Embryo	6.935 (7)	3.50 (7)
	Lymphoma versus Placenta	9.513 (7)	3.50 (7)
	Spleen versus Embryo	11.502 (5)	4.03 (5)
	Spleen versus Placenta	12.193 (3)	5.84 (3)
	Placenta versus Embryo	-4.988 (4)	4.60 (4)
L-phenylalanine Inhibition	Lymphoma versus Spleen	-0.532 (3)	5.84 (3)
	Lymphoma versus Embryo	1.301 (5)	4.03 (5)
	Lymphoma versus Placenta	-0.884 (4)	4.60 (4)
	Spleen versus Embryo	1.279 (5)	4.03 (5)
	Spleen versus Placenta	-0.083 (5)	4.03 (5)
	Placenta versus Embryo	1.760 (6)	3.71 (6)
Magnesium 10.0 mM Activation	Lymphoma versus Spleen	0.728 (8)	3.36 (8)

TABLE I (Continued)

Characteristic	Tissues compared	Calculated t value	t Value for p=0.01*
	Lymphoma versus Embryo	-0.035 (10)	3.17 (10)
	Lymphoma versus Placenta	0.507 (11)	3.11 (11)
	Spleen versus Embryo	-0.993 (4)	4.60 (4)
	Spleen versus Placenta	-0.195 (6)	3.71 (6)
	Placenta versus Embryo	-0.602 (6)	3.71 (6)
Magnesium 5.0 mM Activation	Lymphoma versus Spleen	1.159 (10)	3.17 (10)
	Lymphoma versus Embryo	-0.522 (10)	3.17 (10)
	Lymphoma versus Placenta	0.383 (8)	3.36 (8)
	Spleen versus Embryo	-1.907 (6)	3.71 (6)
	Spleen versus Placenta	-0.488 (5)	4.03 (5)
	Placenta versus Embryo	-0.819 (6)	3.71 (6)
EDTA 1.0 mM Inhibition	Lymphoma versus Spleen	0.506 (7)	3.50 (7)
	Lymphoma versus Embryo	0.931 (6)	3.71 (6)
	Lymphoma versus Placenta	0.892 (6)	3.71 (6)

TABLE I (Continued)

Characteristic	Tissues compared	Calculated t value	t Value for p=0.01*
	Spleen versus Embryo	0.771 (2)	9.92 (2)
	Spleen versus Placenta	0.729 (3)	5.84 (3)
	Placenta versus Embryo	-0.083 (7)	3.50 (7)
EDTA 0.1 mM Inhibition	Lymphoma versus Spleen	1.869 (7)	3.50 (7)
	Lymphoma versus Embryo	2.779 (8)	3.36 (8)
	Lymphoma versus Placenta	3.257 (6)	3.71 (6)
	Spleen versus Embryo	0.680 (6)	3.71 (6)
	Spleen versus Placenta	1.647 (7)	3.50 (7)
	Placenta versus Embryo	-1.162 (6)	3.71 (6)
EDTA 1.0 mM Inhibition	Lymphoma versus Spleen	1.733 (8)	3.36 (8)
	Lymphoma versus Embryo	2.901 (3)	5.84 (3)
	Lymphoma versus Placenta	2.317 (5)	4.03 (5)
	Spleen versus Embryo	2.160 (3)	5.84 (3)
	Spleen versus Placenta	1.376 (5)	4.03 (5)

TABLE I (Continued)

Characteristic	Tissues compared	Calculated t value	t Value for p=0.01*
	Placenta versus Embryo	0.899 (6)	3.71 (6)
EDTA 1.0 mM Inhibition	Lymphoma versus Spleen	1.107 (4)	4.60 (4)
reversed by 5.0 mM	Lymphoma versus Embryo	4.070 (5)	4.03 (5)
Magnesium	Lymphoma versus Placenta	2.368 (4)	4.60 (4)
	Spleen versus Embryo	2.003 (6)	3.71 (6)
	Spleen versus Placenta	1.347 (7)	3.50 (7)
	Placenta versus Embryo	0.130 (6)	3.71 (6)
EDTA 0.1 mM Inhibition	Lymphoma versus Spleen	1.796 (10)	3.17 (10)
reversed by 10.0 mM	Lymphoma versus Embryo	2.291 (4)	4.60 (4)
Magnesium	Lymphoma versus Placenta	3.404 (7)	3.50 (7)
	Spleen versus Embryo	1.291 (3)	5.84 (3)
	Spleen versus Placenta	2.409 (5)	4.03 (5)
	Placenta versus Embryo	-0.743 (7)	3.50 (7)

TABLE I (Continued)

Characteristic	Tissues compared	Calculated t value	t Value for p=0.01*
EDTA 0.1 mM Inhibition reversed by 5.0 mM Magnesium	Lymphoma versus Spleen	2.016 (10)	3.17 (10)
	Lymphoma versus Embryo	1.170 (5)	4.03 (5)
	Lymphoma versus Placenta	1.764 (4)	4.60 (4)
	Spleen versus Embryo	-0.135 (4)	4.60 (4)
	Spleen versus Placenta	1.141 (3)	5.84 (3)
	Placenta versus Embryo	-1.127 (5)	4.03 (5)

Fig. 3. Inhibitory effect of L-phenylalanine on the alkaline phosphatase activity of the leukemic tissues and the normal tissues. H-117 was a spontaneous tumor; U-1 and H-98 were viral induced tumors. The normal tissues included were adult spleen (S), 16-day embryo (E) and 16-day placenta (P). L-phenylalanine was used in 10.0 mM final concentration. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. Percent inhibition is indicated by the bar. The range of values is shown (⊢⊣).

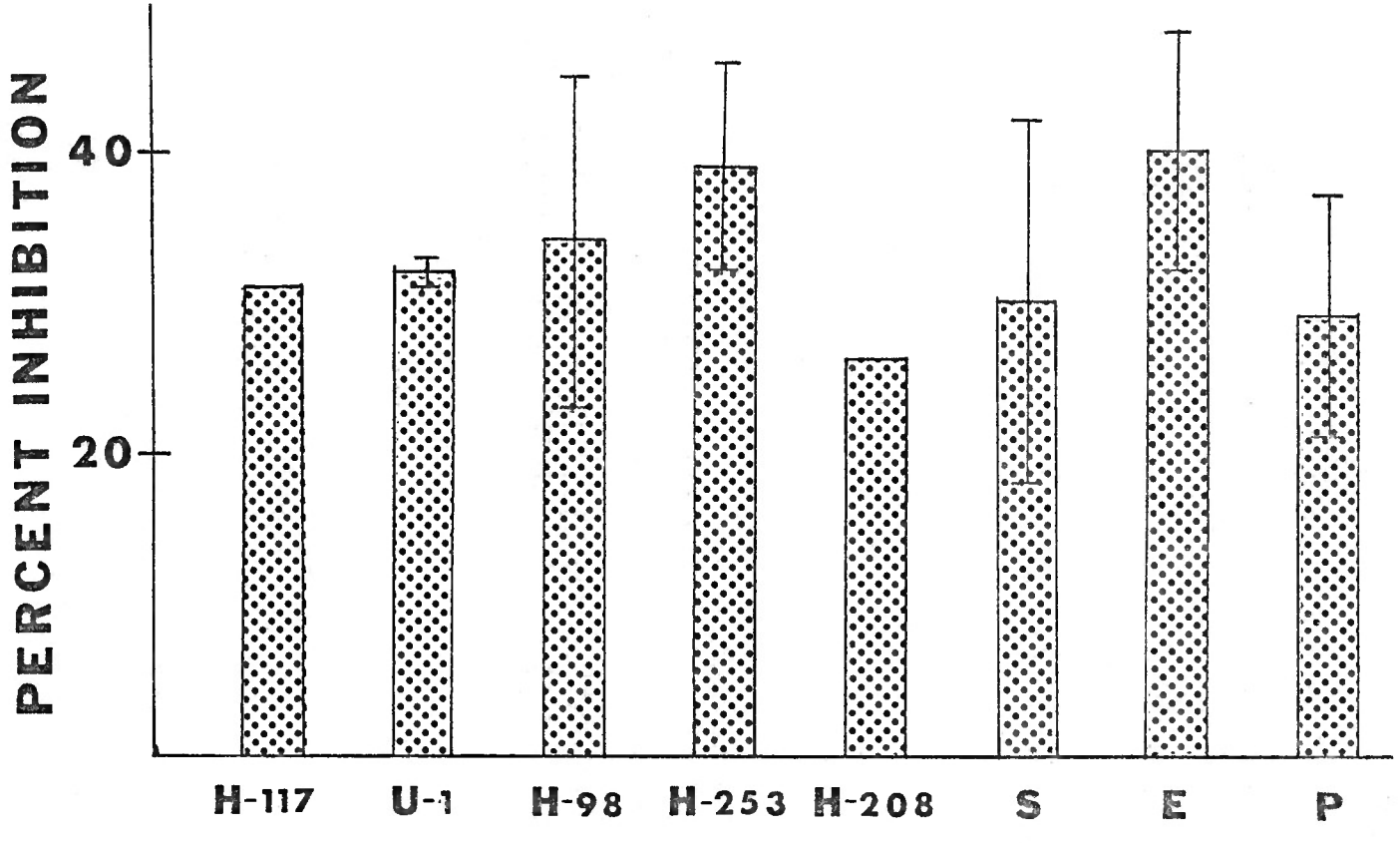

PERCENT INHIBITION
40
20
H-117
U-1
H-98
H-253
H-208
S
E
P

Fig. 4. Activating effect of magnesium ions on the alkaline phosphatase activity of the leukemic tissues. AL represents the average of leukemic values. H-98 and U-1 were virus-induced tumors; H-253 was a cell-transferred tumor; H-117 was a spontaneous tumor; and H-58 was a 6-mercaptopurine-induced tumor. Magnesium ions were used in 5.0 mM and 10.0 mM final concentrations respectively. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. In each tissue bar 1 represents buffer alone; bar 2 represents buffer plus 5.0 mM magnesium chloride; and bar 3 represents buffer plus 10.0 mM magnesium chloride.

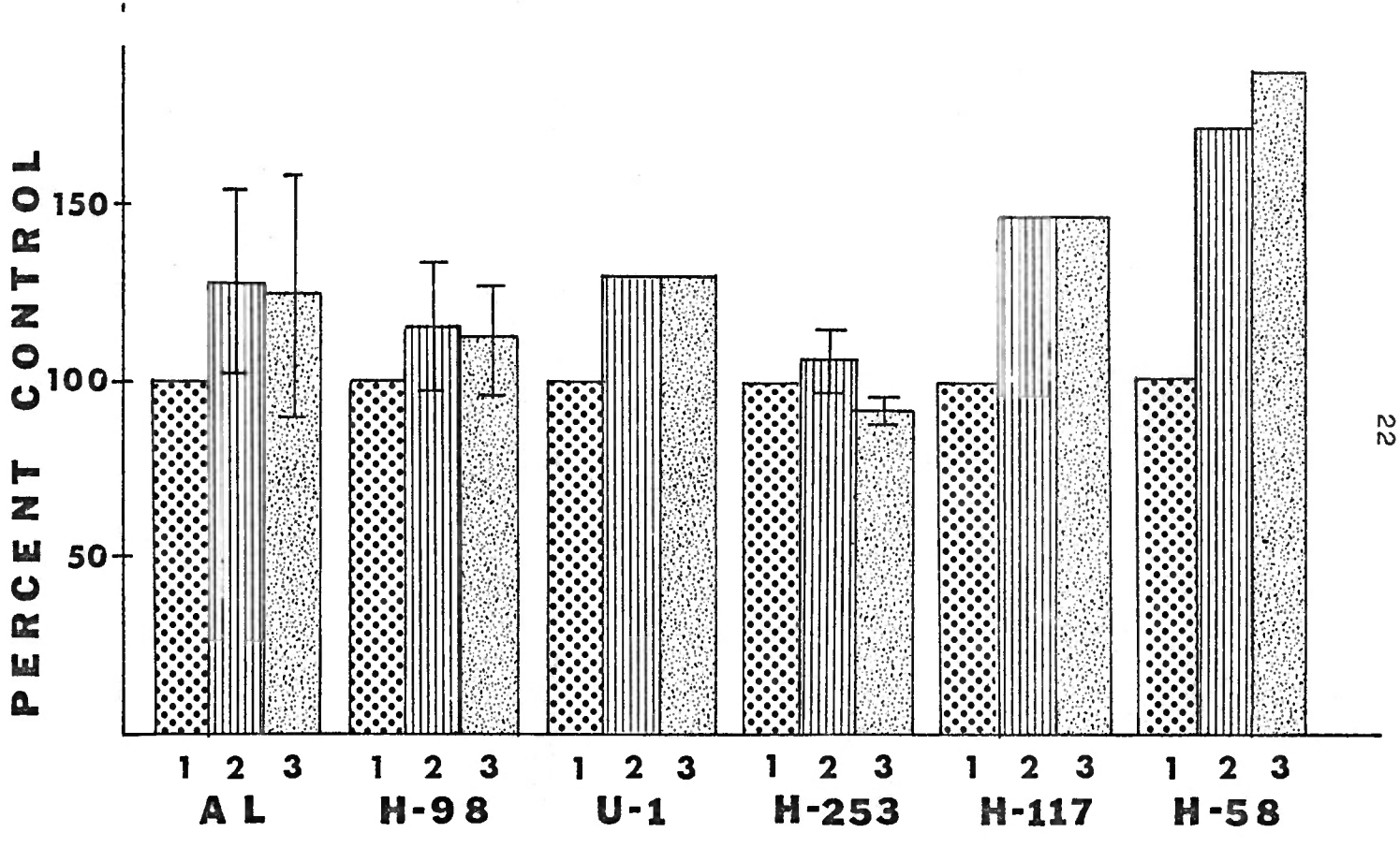

PERCENT CONTROL
150
100
50
1 2 3
AL
H-98
U-1
H-253
H-117
H-58

Figure 5 shows the effect of magnesium ions on normal tissue APase activities compared to the effect on lymphoma APase activity. The normal tissues are activated to different extents with the concentrations of exogeneous magnesium ions being used. Statistically there was no significant difference ($p > 0.01$, Table 1) between the isoenzymes of normal and leukemic tissues according to the extent of their activation.

Magnesium Content of Different Tissue Extracts

Fluorescence spectrophotometry was used in the determination of magnesium content of different tissue extracts (Table 2). These experiments were done to account for endogeneous magnesium ions in the individual tissue extracts to see if this would explain the variability in the activation results. The lymphoma extracts showed higher magnesium content but that did not correlate with activation levels.

Reversal Experiments

Figure 6 shows the inhibitory effect of 0.1 mM EDTA on leukemic tissues and the average of leukemic values. This inhibition is reversed by the addition of magnesium ions in 5.0 mM and 10.0 mM final concentrations. The data in Table 3 show that added magnesium ions have a significant ($p < 0.01$) reversal effect.

Fig. 5. Activating effect of magnesium ions on the alkaline phosphatase activity of the normal tissues as compared to the average of leukemic values. AL represents the average of leukemic values which is the same as used in Fig. 4. The normal tissues included were adult spleen (S), 16-day embryo (E), and 16-day placenta (P). Magnesium ions were used in 5.0 mM and 10.0 mM final concentrations respectively. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. In each tissue bar 1 represents buffer alone; bar 2 represents buffer plus 5.0 mM magnesium chloride and bar 3 represents buffer plus 10.0 mM magnesium chloride.

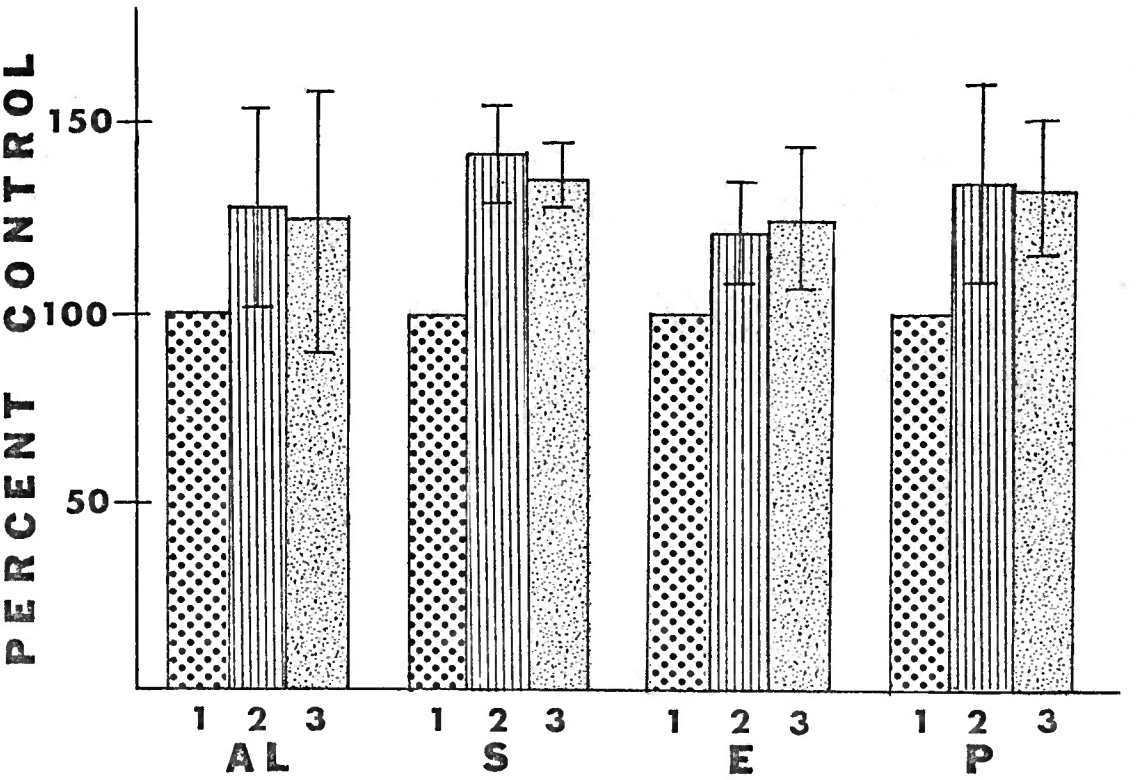

PERCENT CONTROL
150
100
50
1 2 3
AL
1 2 3
S
1 2 3
E
1 2 3
P

TABLE II

Magnesium Content of Different Tissue Extracts

Tissues	Magnesium Concentration x 10^{-6}M
Spleen	0.60
Whole embryo	0.80
Placenta	0.50
U-1	2.00
H-98	1.50
H-253	1.60
H-208	0.80

Fig. 6. Inhibition by 0.1 mM EDTA of the alkaline phosphatase activity of the leukemic tissues and its reversal with magnesium ions. AL represents the average of leukemic values; H-98 and U-1 were virus-induced tumors; H-253 was a cell-transferred tumor; H-117 was a spontaneous tumor; and H-58 was a 6-mercaptopurine-induced tumor. The magnesium ions were used in 5.0 mM and 10.0 mM final concentrations respectively. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM as a substrate. In each tissue bar 1 represents EDTA alone; bar 2 represents EDTA plus 5.0 mM magnesium chloride and bar 3 represents EDTA plus 10.0 mM magnesium chloride.

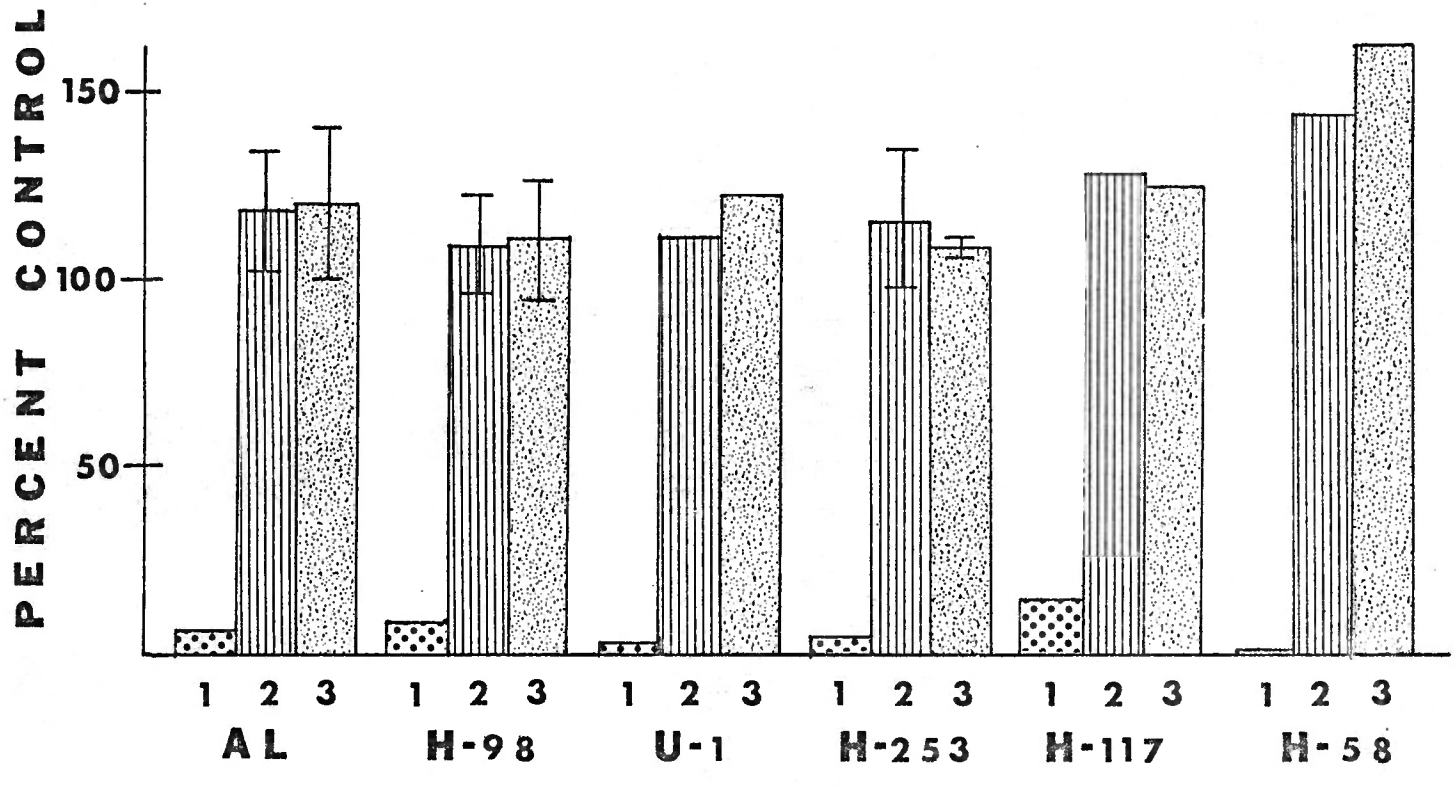

PERCENT CONTROL
150
100
50
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
AL
H-98
U-1
H-253
H-117
H-58

TABLE III

Statistical Significance of the Reversal of the EDTA Inhibited Alkaline Phosphatase Activity of the Leukemic and Normal Tissues by the Addition of Magnesium Chloride

Tissue	Concentration of EDTA in mM	Concentration magnesium in mM	Calculated t value	t Value for p=0.01
Lymphoma	0.1 mM	5.0 mM	18.377 (7)*	3.50 (7)
Lymphoma	0.1 mM	10.0 mM	14.535 (7)	3.50 (7)
Spleen	0.1 mM	5.0 mM	23.670 (4)	4.60 (4)
Spleen	0.1 mM	10.0 mM	20.842 (3)	5.84 (3)
Embryo	0.1 mM	5.0 mM	11.615 (2)	9.92 (2)
Embryo	0.1 mM	10.0 mM	10.160 (2)	9.92 (2)
Placenta	0.1 mM	5.0 mM	6.145 (3)	5.84 (3)
Placenta	0.1 mM	10.0 mM	11.692 (4)	4.60 (4)
Lymphoma	1.0 mM	5.0 mM	11.471 (7)	3.50 (7)
Lymphoma	1.0 mM	10.0 mM	10.471 (6)	3.71 (6)
Spleen	1.0 mM	5.0 mM	6.592 (2)	9.92 (2)
Spleen	1.0 mM	10.0 mM	11.976 (2)	9.92 (2)

TABLE III (Continued)

Tissue	Concentration of EDTA in mM	Concentration magnesium in mM	Calculated t value	t Value for p=0.01
Embryo	1.0 mM	5.0 mM	11.061 (2)	9.92 (2)
Embryo	1.0 mM	10.0 mM	6.055 (2)	9.92 (2)
Placenta	1.0 mM	5.0 mM	6.102 (3)	5.84 (3)
Placenta	1.0 mM	10.0 mM	6.427 (3)	5.84 (3)

* Figures in parentheses ( ) indicate degrees of freedom.

Figure 7 shows the inhibitory effect of 0.1 mM EDTA on the APase activities of normal tissues as compared to the average of leukemic values and the reversal of this inhibition with the addition of magnesium ions in 5.0 mM and 10.0 mM final concentrations. Table 3 also shows that the reversal is significant in the normal tissues. The data in Table 1 show that there is no significant difference between the APase activities of the normal tissues and leukemic tissues according to the extent of their reversal with added magnesium ions ($p > 0.01$).

Figure 8 shows the inhibitory effect of 1.0 mM EDTA on leukemic APases and the average of leukemic values. The inhibition is reversed by 5.0 mM and 10.0 mM magnesium ions. The data in Table 3 show that there is significant reversal by both the concentrations of magnesium ions ($p < 0.01$).

Figure 9 shows the inhibitory effect of 1.0 mM EDTA concentration on the normal tissues and the average of leukemic values and its reversal with added magnesium ions at 5.0 mM and 10.0 mM final concentrations. The reversal effect is significant in all four tissues ($p < 0.01$, Table 3). As shown in Table 1 there is no significant difference between the normal tissue APase activities and the leukemic tissue APase activities according to the extent of their reversal with added magnesium ions ($p > 0.01$).

Fig. 7. Inhibition by 0.1 mM EDTA of the alkaline phosphatase activity of the normal tissues as compared to the average of leukemic values and its reversal with magnesium ions. AL represents the average of leukemic values which is the same as used in Fig. 6. The normal tissues included were adult spleen (S), 16-day embryo (E) and 16-day placenta (P). The magnesium ions were used in 5.0 mM and 10.0 mM final concentrations respectively. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. In each tissue bar 1 represents EDTA alone; bar 2 represents EDTA plus 5.0 mM magnesium chloride bar; and 3 represents EDTA plus 10.0 mM magnesium chloride.

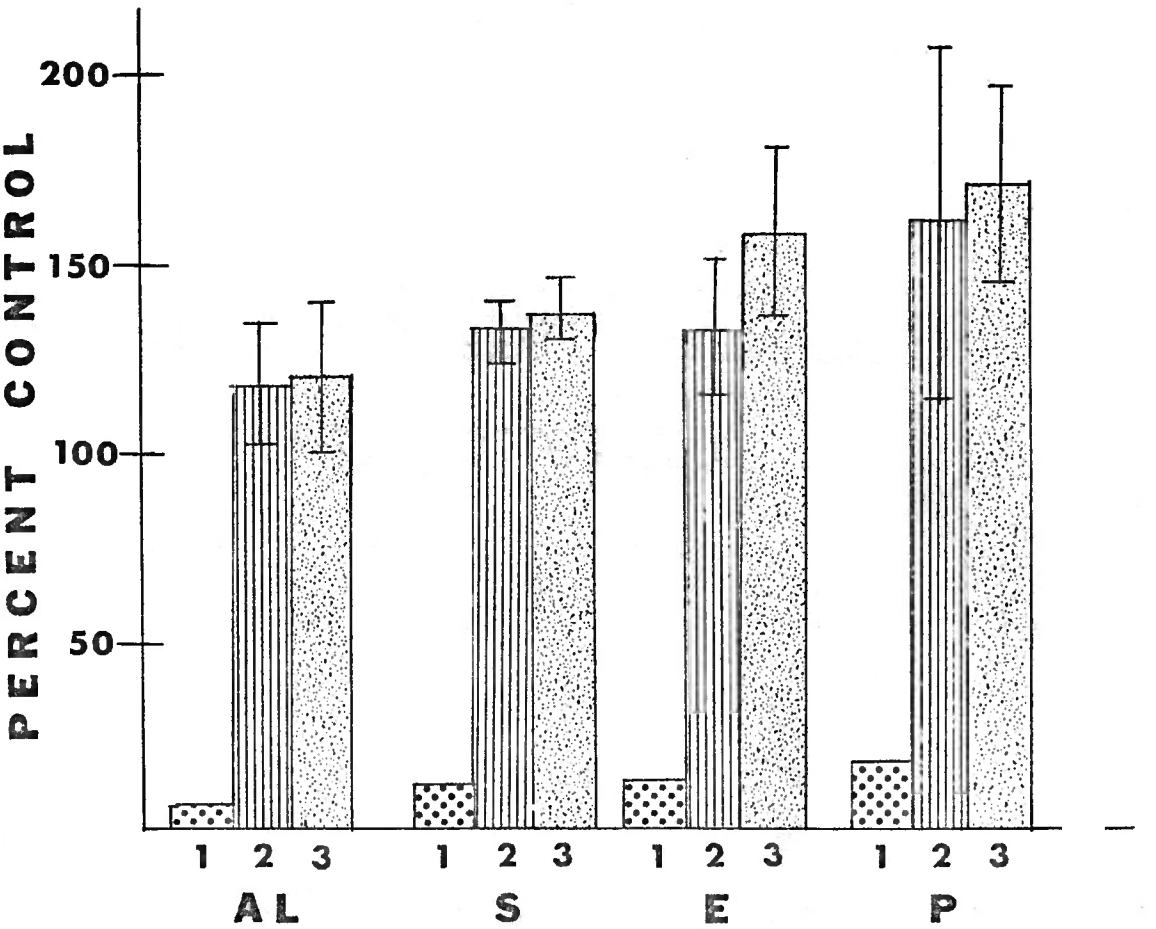

PERCENT CONTROL
200
150
100
50
1 2 3
1 2 3
1 2 3
1 2 3
AL
S
E
P

Fig. 8. Inhibition by 1.0 mM EDTA of the alkaline phosphatase activity of the leukemic tissues and its reversal with magnesium ions. AL represents the average of leukemic values; H-98 and U-1 were virus-induced tumors., H-253 was a cell-transferred tumor; H-117 was a spontaneous tumor; and H-58 was a 6-mercaptopurine-induced tumor. The magnesium ions were used in 5.0 mM and 10.0 mM final concentrations respectively. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. In each tissue bar 1 represents EDTA alone; bar 2 represents EDTA plus 5.0 mM magnesium chloride; and bar 3 represents EDTA plus 10.0 mM magnesium chloride.

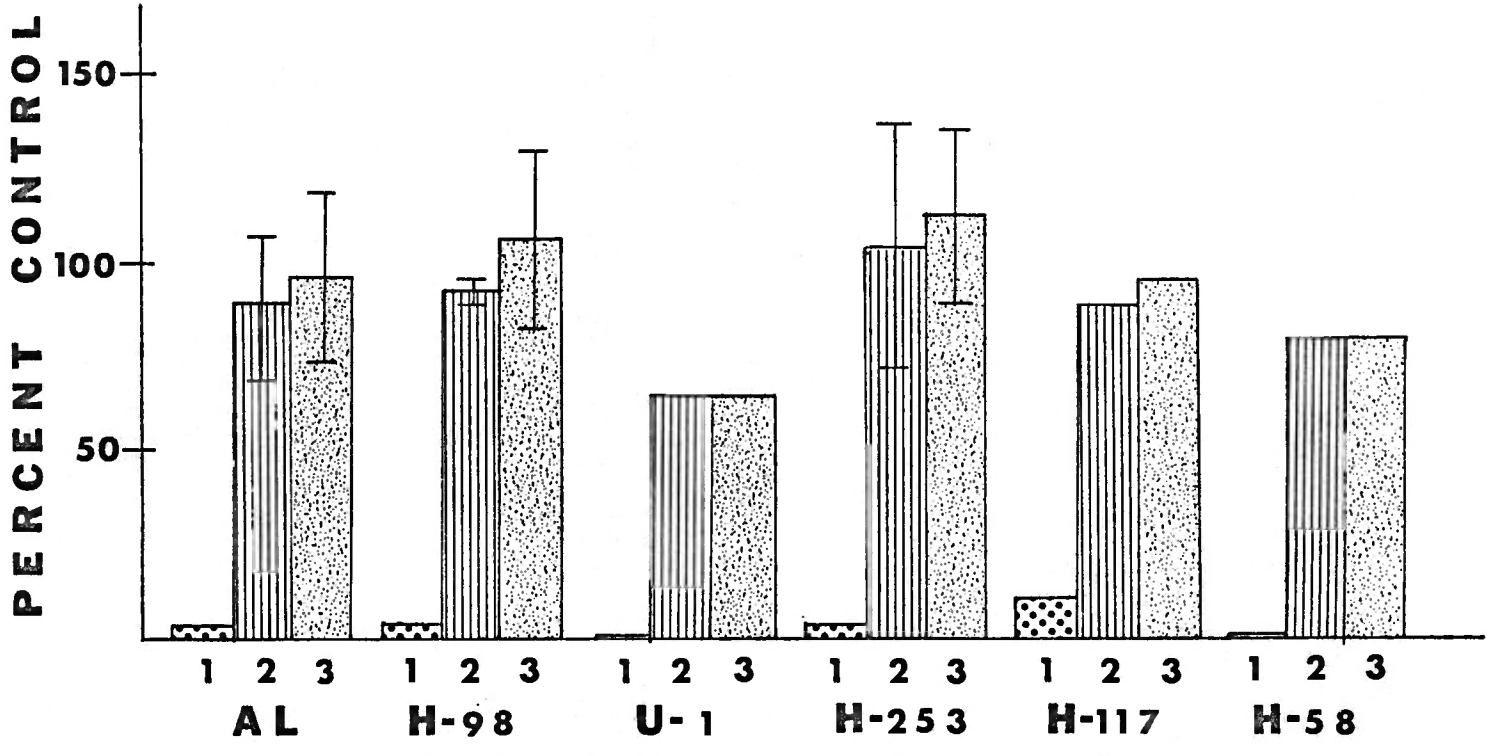
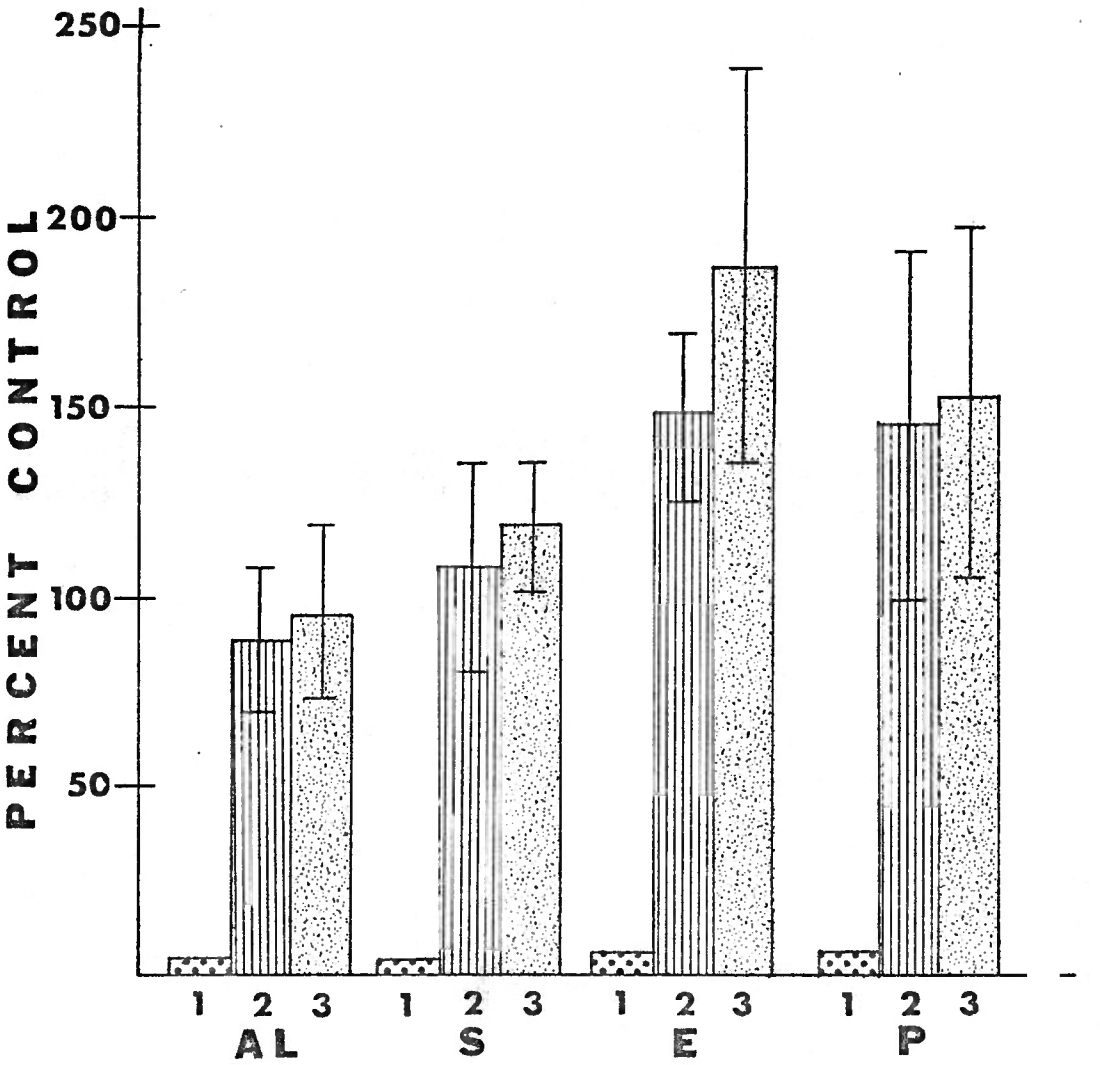

PERCENT CONTROL
150
100
50
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
AL
H-98
U-1
H-253
H-117
H-58

Fig. 9. Inhibition by 1.0 mM EDTA of the alkaline phosphatase activity of the normal tissues as compared to the average of leukemic values and its reversal with magnesium ions. AL represents the average of leukemic values which is the same as used in Fig. 8. The normal tissues included were adult spleen (S), 16-day embryo (E) and 16-day placenta (P). The magnesium ions were used in 5.0 mM and 10.0 mM final concentrations respectively. The experiments were performed at 37 C for 30 minutes with pNPP in a final concentration of 3.38 mM. In each tissue bar 1 represents EDTA alone; bar 2 represents EDTA plus 5.0 mM magnesium chloride; and bar 3 represents EDTA plus 10.0 mM magnesium chloride.


PERCENT CONTROL
250
200
150
100
50
1 2 3 1 2 3 1 2 3 1 2 3
AL
S
E
P

CHAPTER V

DISCUSSION AND CONCLUSIONS

Inhibitors have been used extensively in the identification of isoenzymes of APase from different organ sources. In the present study zinc sulfate, L-phenylalanine and EDTA were used as inhibitors of APase isoenzymes from normal and leukemic tissues of C57Bl mice. The results with zinc sulfate were particularly significant and gave 32% and 56% inhibition of APase activity of whole embryo and placenta, respectively; the spleen and leukemic tissue APase activities were not affected. L-phenylalanine partially inhibited normal and leukemic APase activities and did not show any preferential inhibition as reported by Fishman, et al. (1962) for rat intestine APase. EDTA in both the concentrations being used in these experiments (0.1 mM and 1 mM) was inhibitory for APase activities of both normal and leukemic tissues. This inhibition was reversed with the exogenous addition of magnesium ions at 5.0 mM and 10.0 mM concentrations.

Abul-Fadl and King (1949) and Hoare and Delory (1955) have shown that APase from ox kidney and sodium cyanide-treated ox kidney APases are inhibited by zinc sulfate. Fishman et al. (1962) have shown that APase from bone, lung, liver, and kidney are inhibited by zinc while intestinal and blood serum APases are not. The results of Clark and

Porteous (1965) showed that zinc is a powerful inhibitor of APase from the rabbit small intestine. According to Hoare and Delory (1955) the exogenous zinc ions lead to inhibition because of their non-specific effect on proteins.

L-phenylalanine has been used by Fishman et al. (1963) as an organ specific, stereospecific inhibitor of human and rat intestinal APase isoenzymes. Our results correspond with Brunel and Cathala (1971) who obtained 38% inhibition of beef brain APase with L-phenylalanine and with Belle (1972) in the sense that he could not distinguish APase isoenzymes from different canine tissues on the basis of L-phenylalanine inhibition. Eaton and Moss (1967) reported that L-phenylalanine inhibits orthophosphatase and pyrophosphatase activities of human intestinal APase but does not affect either activity of the liver enzyme. Floyd (1973) has shown that in C57Bl mice orthophosphatase and pyrophosphatase activities reside in different molecules.

EDTA has been used extensively as an inhibitor of APase from different sources. Trubowitz et al. (1957) reported that EDTA inhibited APase activity from human leukocytes. Mathies (1958) used EDTA to show the inhibition of his purified swine kidney APase. Clark and Porteous (1965) reported that EDTA dialyzed APase can not be activated with zinc or magnesium ions alone but both must be present and especially zinc should not exceed an optimum limit.

Agus et al. (1966) reported the cysteine inhibition of human kidney APase. According to his results cysteine inhibition can be reversed by dialysis or passage through coarse grade sephadex, while EDTA inhibition requires the readdition of zinc or magnesium ions. These results correspond with our work as far as reversal of EDTA inhibition with magnesium ions are concerned.

Support for our results with magnesium ion activation of APase from normal and leukemic tissues come from the work of Fischer and Greep (1948) who have reported the magnesium ion activation of intestinal APase. Abul-Fadl and King (1949) showed that magnesium ions activate kidney APase. Hoare and Delory (1955) working with ox kidney APase reported the activation of cyanide-treated APase with magnesium ion. Fishman et al. (1962) reported magnesium ion activation of bone, kidney, lung, blood, intestine and liver APases. Eaton and Moss (1967, 1968) have reported that magnesium ions activate the orthophosphatase activity but inhibit pyrophosphatase activity except in low concentration. This was true for human intestine, liver and bone APases.

Lagerlöf and Kaplan (1967) have demonstrated APase in the thymus of 16-day-old embryos by the use of histochemical methods. It disappears after this time period and then reappears in the thymic lymphomas. This observation, along

with the biochemical characterization of APase from normal and leukemic tissues by Lumb and Doell (1970), suggests that APase appears in the thymic lymphomas by the derepression of embryonic genes. Our zinc sulfate inhibition results support the observations of Lumb and Doell (1970).

Derepression of the embryonic antigens in cancer and by tumor viruses have been reported in literature repeatedly. Gold and Freeman (1965) reported the presence of specific carcinoembryonic antigens in human colon carcinoma. Kithier et al. (1966) have reported the appearance of fetal serum protein in the serum of primary liver carcinoma-bearing patients and Stanislawski-Birencwajg et al. (1967) demonstrated fetal serum proteins in the serum of chemically induced primary liver carcinoma in rats. Hull et al. (1969) demonstrated alpha-fetoproteins in the serum of monkeys suffering from liver carcinomas. Pearson and Freeman (1968) have implicated polyoma viruses in the derepression phenomenon leading to the appearance of an embryonic antigen.

Recently Ambrose et al. (1971) have shown the resemblance between tumor specific transplantation antigens (TSTA) produced by SV40 virus infection and the embryonic antigens. They have shown that neonatally SV40-infected hamster can be immunized against tumor formation with human embryo or hamster embryo cells. They have argued that

during embryonic life there is much less difference among the proteins of different animals if the concept that ontogeny repeats phylogeny is applicable at the molecular level. The immunization effect of fetal antigens indicate that tumor specific transplantation antigens appear as a result of derepression by tumor viruses.

The differentiating zinc sulfate inhibition results have shown that APase from the leukemic tissues resemble that from the normal adult spleen. These results in light of those obtained by Lumb and Doell (1970) support their suggestion that APase in mouse lymphoma cells appears as a result of derepression of embryonic functions.

CHAPTER VI

SUMMARY

1. Magnesium ions activate the APase activity of normal as well as leukemic tissues. No significant difference is found between the normal and leukemic tissue APases according to the extent of their activation by magnesium.
2. EDTA is the strongest inhibitor of APase activity both from the normal and the leukemic tissues.
3. L-phenylalanine partially inhibits the APase activity from all the tissues tested, but does not have a differentiating effect.
4. Zinc sulfate has a differentiating inhibitory effect. It inhibits embryonic and placental APase activity, but does not inhibit spleenic and leukemic APases.
5. Magnesium ions reverse the inhibitory effect of EDTA on APase activity in all the tissues tested.
6. The experimental results of zinc sulfate inhibition studies have shown that normal adult spleen APase resembles the thymic lymphoma APase. These results in light of those obtained by Lumb and Doell (1970) support their suggestion that APase in thymic lymphoma is formed as a result of the derepression of embryonic functions.

LITERATURE CITED

Abul-Fadl, M. A., and King, E. J. 1949. Reactivation of alkaline phosphatase after dialysis. Biochem. J. 44: 435-441.

Agus, S. G., Cox, R. P., and Griffin, M. J. 1966. Inhibition of alkaline phosphatase by cysteine and its analogues. Biochem. Biophys. Acta 118: 363-370.

Ambrose, K. R., Anderson, N. G., and Coggin, J. H. 1971. Interruption of SV40 oncogenesis with human foetal antigen. Nature 233: 194-195.

Belle, H. V. 1972. Kinetics and inhibition of alkaline phosphatase from canine tissues. Biochem. Biophys. Acta 289: 158-168.

Bodansky, O. 1937. Are the phosphatases of bone, kidney, intestine and serum identical? The use of bile acids in their differentiation. J. Biol. Chem. 118: 341-362.

Brunel, C., Cathala, G., and Saintot, M. 1969. Purification and properties of alkaline phosphatase from beef brain. (In French, English summary) Biochem. Biophys. Acta 191: 621-635.

Brunel, C., and Cathala, G. 1971. Beef brain alkaline phosphatase: Role of magnesium on the action of L-phenylalanine and other amino acids. (In French, English summary) Biochem. Biophys. Acta 235: 106-118.

Clark, B., and Porteous, J. W. 1965. The metal ion activation of the alkaline beta-glycerophosphatase of rabbit small intestine. Biochem. J. 95: 475-482.

Cloetens, R., 1939. Identification de deux, phosphatases alcalines dans les organes animaux. Enzymologia. 6: 46-56.

Cox, R. P., and Griffin, M. J. 1967. Alkaline phosphatase: Comparison of the physical and chemical properties of enzymes preparations from mamalian cell cultures, various animal tissues and Escherichia coli. Arch. Biochem. Biophys. 122: 552-562.

Doell, R. G., and Mathieson, B. J. 1970. Absence of alkaline phosphatase in rat thymic lymphoma induced by murine leukemia virus. Cancer Res. 30: 2456-2457.

Doell, R. G., and Mathieson, B. J. 1971. Serological studies of tumor antigens in chemical-and virus-induced thymic lymphomas. Cancer Res. 31: 1285-1289.

Drill, V. A., and Riggs, D. S. 1946. Inhibition of alkaline serum phosphatase activity during liver disease. J. Biol. Chem. 162: 21-25.

Eaton, R. H., and Moss, D. W. 1967. Inhibition of the orthophosphatase and pyrophosphatase activities of human alkaline phosphatase preparations. Biochem. J. 102: 917-921.

Eaton, R. H., and Moss, D. W. 1968. Partial purification and some properties of human bone alkaline phosphatase. Enzymologia 35: 31-39.

Ferrer, J. F., and Kaplan, H. S. 1968. Antigenic characteristics of lymphomas induced by radiation leukemia virus (RadLV) in mice and rats. Cancer Res. 28: 2522-2528.

Fischer, C. J., and Greep, R. O. 1948. Studies on activation of purified alkaline phosphatase. Arch. Biochem. 16: 199-219.

Fishman, W. H., Green, S., and Inglis, N.I. 1962. Organ specific behavior exhibited by rat intestine and liver alkaline phosphatase. Biochem. Biophys. Acta 62: 363-375.

Fishman, W. H., Green, S., and Inglis, N. I. 1963. L-phenylalanine an organ specific, stereospecific inhibitor of human intestinal alkaline phosphatase. Nature 198: 685-686.

Floyd, R. A. 1973. A comparative electrophoretic study of normal and leukemic tissues. M. S. Thesis, Atlanta University.

Gold, P., and Freeman, S. O. 1965. Specific carcinoembryonic antigens of the human digestive system. J. Exptl. Med. 122: 467-481.

Goldstein, A. 1964. Biostatistics: An introductory text. The Macmillan Company, New York.

Gross, L. 1951. Spontaneous leukemia developing in C3H mice following inoculation in infancy with Ak-leukemic extracts or AK-embryos. Proc. Soc. Exptl. Biol. Med. 76: 27-32.

Haran-Ghera, N. 1967. A leukemogenic filterable agent from chemically-induced lymphoid leukemia in C57Bl mice. Proc. Soc. Exptl. Biol. Med. 124: 697-699.

Hoare, R., and Delory, G. E. 1955. The effect of zinc and magnesium ions on the activity of kidney alkaline phosphatase. Arch. Biochem. Biophys. 59: 465-472.

Hull, E. W., Carbone, P. P., Gitlin, D., O'gara, R. W., and Kelly, M. G. 1969. Alpha-fetoprotein in monkeys with hepatoma. J. Natl. Cancer Inst. 42: 1035-1040.

Kaplan, H. S., and Brown, M. B. 1952. Protection against radiation induced lymphoma development by shielding and partial-body irradiation of mice. Cancer Res. 12: 441-444.

Kithier, K., Houstek, J., Masopust, J., and Radl, J. 1966. Occurrence of a specific foetal protein in a primary liver carcinoma. Nature 212: 414.

Lagerlöf, B. A. M., and Kaplan, H. S. 1967. Specificity of the relationships between thymic alkaline phosphatase

activity and lymphoma development in strain C57Bl mice. J. Natl. Cancer Inst. 38: 437-458.

Lieberman, M., and Kaplan, H. S. 1959. Leukemogenic activity of filtrates from radiation-induced lymphoid tumors of mice. Science 130: 387-388.

Lumb, J. R., and Doell, R. G. 1970. The biochemical characterization of alkaline phosphatase from chemical- and viral-induced thymic lymphomas of C57Bl mice. Cancer Res. 30: 1391-1396.

Mathies, J. C. 1958. Preparation and properties of highly purified alkaline phosphatase from swine kidney. J. Biol. Chem. 233: 1121-1129.

Metcalf, D., Sparrow, N., and Wyllie, R. 1962. Alkaline phosphatase activity in mouse lymphoma tissue. Australian J. Exptl. Biol. Med. Sci. 40: 215-224.

Pearson, G., and Freeman, G. 1968. Evidence suggesting a relationship between polyoma virus-induced transplantation antigen and normal embryonic antigen. Cancer Res. 28: 1665-1673.

Rufo, M. B., and Fishman, W. H. 1972. L-Homoarginine, a specific inhibitor of liver type alkaline phosphatase applied to the recognition of liver type enzyme activity in rat intestine. J. Histochem. Cytochem. 20: 336-343.

Schachter, D. 1959. The fluorometric estimation of magnesium in serum and in urine. J. Lab. Clin. Med. 54: 763

Schachter, D. 1961. Fluorometric estimation of magnesium with i-Hydroxy-5-Quinoline-sulfonate. J. Lab. Clin. Med. 58: 495

Siegler, R., and Rich, M. A. 1967. Significance of increased alkaline phosphatase activity in viral-induced thymic lymphoma. Proc. Soc. Exptl. Biol. Med. 125: 868-871.

Smith, C. 1961. Studies on the thymus of the mammal. XII. Histochemistry of the thymuses of C57Bl/6 and AKR strain mice. J. Natl. Cancer Inst. 26: 389-404.

_____ 1962. Studies on the thymus of the mammal. XIII. Histochemistry of irradiated thymuses of C57Bl strain mice. J. Natl. Cancer Inst. 29: 375-387.

Stanislawski-Birencwajg, M., Uriel, J., and Grabar, P. 1967. Association of embryonic antigens with experimentally induced hepatic lesions in the rat. Cancer Res. 27: 1990-1997.

Trubowitz, S., Feldman, D., Benante, C., and Kirman, D. 1957. Metal requirements of alkaline phosphatase of human and rabbit leukocytes. Proc. Soc. Exptl. Biol. Med. 95: 35-38.

Williams, M. T. 1971. Studies on inhibition, activation and substrate specificity of the alkaline phosphatase activity from the thymic lymphomas of C57Bl mice. M. S. Thesis, Atlanta University.